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Original Article

TROP-2, NECTIN-4 and predictive biomarkers in sarcomatoid and rhabdoid bladder urothelial carcinoma

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Summary

Introduction. The surface protein TROP-2/*TACSTD2* and the cell adhesion protein NECTIN-4/*NECTIN4* are responsible for the efficacy of anticancer therapies based on antibody-drug conjugates (ADC) targeting intracellular microtubules. In contrast with common histologic subtypes of bladder urothelial carcinoma (BUC), little is known of TROP-2 and NECTIN-4 expression in sarcomatoid and rhabdoid BUC.

Aims. In this study, we aimed to analyze TROP-2 and NECTIN-4 expression and additional predictive biomarkers by immunohistochemistry and fluorescence in situ hybridization (FISH) on 35 undifferentiated BUC (28 sarcomatoid and 7 rhabdoid). Wide genomic investigation was also performed on 411 BUC cases of the PanCancer Atlas, focusing on genes related to the microtubule pathways.

Results. Seven of 35 (20%) undifferentiated BUC showed expression of TROP-2. NECTIN-4 was expressed in 10 cases (29%). Seven cases (20%) co-expressed TROP-2 and NECTIN-4. *HER-2* FISH was amplified in 5 cases (14%) while HER-2 immunoexpression was observed in 14 cases (40%). PD-L1 scored positive for combined proportion score (CPS) in 66% of cases and for tumor proportion score (TPS) in 51% of cases. Pan-NTRK1-2/3 was elevated in 9 cases (26%) and *FGFR-2/3* was broken in 7 of 35 cases (20%). Of 28 sarcomatoid BUC, 9 (32%) were negative for all (TROP-2, NECTIN-4, PD-L1, *HER-2*, *FGFR* and pan-NTRK) biomarkers and 3 (11%) expressed all five biomarkers. Among cases with rhabdoid dedifferentiation, 1 of 7 (14%) showed activation of all biomarkers, whereas 2 of 7 (28%) showed none. The mRNA analysis identified microtubule-related genes and pathways suitable for combined ADC treatments in BUC.

Conclusion. Sarcomatoid and rhabdoid BUC do harbor positive expression of the ADC targets TROP-2 or NECTIN-4 in a relatively modest subset of cases, whereas the majority do not. Different combinations of other positive biomarkers may help the choice of medical therapies. Overall, these findings have important clinical implications for targeted therapy for BUC.

Key words: bladder, biomarker, TROP-2, NECTIN-4, urothelial carcinoma

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Introduction

Patients affected by locally advanced bladder urothelial carcinoma (BUC) or with distant metastases may benefit from a variety of available medical therapies among three clinical scenarios (neoadjuvant, adjuvant or metastatic setting) [1]. Recently, new generation targeted therapies, including drugs with strong activation against the microtubule pathways, have been approved [2]. The antibody-drug conjugate (ADC) Enfortumab-vedotin mainly acts by targeting the NECTIN-4 molecule, a microtubule protein that is ubiquitously expressed in conventional bladder carcinoma [2]. Similarly, anti-TROP-2 ADC drugs were shown to harbor efficacy on metastatic urothelial carcinoma by targeting microtubules in the epithelial cells of bladder carcinoma [2]. The phase 2 trial of sacituzumab govitecan (TROPHY-U-01, NCT03547973) in patients with pretreated, locally advanced, or metastatic bladder urothelial carcinomas has shown a significant overall response rate [3]. Additionally, combinations of sacituzumab govitecan with other drugs, including with the NECTIN-4-targeting ADC enfortumab-vedotin (NCT04724018), also showed promising efficacy [4]. Even more recently, enfortumab plus pembrolizumab showed a high confirmed objective response with durable responses using a first-line treatment in cisplatin-ineligible patients with locally advanced or metastatic urothelial carcinoma [5].

At the tissue level, the surface protein TROP-2, encoded by the gene TACSTD2/TROP-2, is a calcium signaling transmembrane protein, whereas NECTIN-4 is a single-pass type I membrane protein [2,4]; both proteins are related to the microtubule and explain the efficacy of targeted therapies, with microtubules being the direct targets [4]. At the cellular level, the efficacy of the aforementioned drugs is related to the heavy presence of crowded proteins in the cytoplasm of the neoplastic cells [2,4]. The conventional subtype of bladder carcinoma has already been characterized for the presence of both proteins [2,4]. Unlike conventional morphological subtype, little is known regarding their expression in undifferentiated urothelial carcinoma, specifically tumors with sarcomatoid or rhabdoid histological phenotypes [6]. We aimed to study a series of undifferentiated BUC for the expression of TROP-2 and NECTIN-4 to provide the rationale for the use of the above-mentioned therapies in these special types of tumors. Furthermore, we simultaneously matched such molecule expression to other tissue-targeted biomarkers, including PD-L1, *FGFR2/3*, *HER-2*/HER-2, and pan-NTRK pathways. Genomic investigation was also performed on the BUC cases of the PanCancer Atlas (TCGA), focusing on 100 genes related to the microtubule pathway and 200 genes whose expression correlated with that of both *TACSTD2* and *NECTIN4*. This analysis was performed in order to explore additional molecules of interest related to the aforementioned pathways with the aim of investigating the prevalence of these potential molecular abnormalities in international public cohorts.

Materials and methods

Samples, Immunostaining and FISH Analysis

Tissue collection, immunohistochemical, and FISH studies were performed as detailed in Figure 1. From a multicentric study cohort, tissues related to muscle-infiltrating urothelial carcinomas from complete bladder resection (biopsy excluded per the current study) were centrally reviewed by three expert uropathologists (MB, GM, SG). Only cases demonstrating a pure undifferentiated morphology were selected. Thirty-five cases of undifferentiated BUC were recruited and included in the study (approved by the ethics committee procedure code PRIHTA-2014-00000453). Twenty-eight sarcomatoid subtypes and seven rhabdoid subtypes were recognized among these cases.

A single representative paraffin block per case was selected for immunohistochemical analysis. A positive value was scored if at least 5% of neoplastic cells expressed TROP-2 (Invitrogen, clone: TACTD2/2152; dilution 1:5000). Staining for NECTIN-4 (Abcam, clone: EPR15613-68; dilution 1:1000) was scored for intensity (0-3) and extent (% positive cells) using the H-score system, where > 15 was considered positive. HER-2 was scored according to HercepTest (Ventana, Roche) (score 0-1-2 and 3+). Pan-NTRK (Abcam, clone: EPR17341; dilution 1:50) was scored when at least 5% of neoplastic cells showed immunoexpression. PD-L1 was evaluated and scored using the tumor proportion score (TPS) and combined proportion score (CPS) for the Ventana-Roche kit (clone SP263) and using CPS for Dako Autostainer kit (clone 22C3).

FISH analysis on tissue was evaluated using the *HER-2* FISH kit (Leica Systems). Amplification was registered when the ratio between *HER-2*/CEP17 was ≥2 with an absolute number of *HER-2* > 6, and following the ASCO/CAP 2018 guidelines. FISH analysis was also performed for the locus specific probe *FGFR-2/3* using Leica Systems. Gains and losses were reported when at least 10% of neoplastic nuclei harbored numerical anomalies.

Images were digitalized using the Grundium Ocus scanner and statistical analysis was performed to evaluate mutual or simultaneous immunohistochemical or molecular abnormalities.

Figure 1. Heat map for TROP-2, NECTIN-4 and other biomarkers in 35 undifferentiated urothelial bladder carcinoma (Red: positive; Green; negative).

Transcriptomic investigation

Z-scores relative to diploid samples (RNA Seq V2 RSEM) of 100 microtubule genes in addition to *NECTIN4* and *TACSTD2* of 411 Bladder Urothelial Carcinoma (BUC) cases was extracted from the TCGA, PanCancer Atlas study by cBioPortal (accessed November 2022) [7]. The clustering function included in cBioPortal applied to mRNA levels identified 3 sample groups (sample group 1A, 1B and 2) characterized by related expression patterns and defining 2 gene clusters (*gene cluster 1* and *gene cluster 2*). We then extended the transcriptomic analysis of the microtubule pathway to the entire cellular context by extracting *NECTIN4*-related gene expression from RNAseq data by selecting the top 200 genes positively related to *NECTIN4* ($r > 0.5$, adjusted $P < 0.00002$). For the gene enrichment analysis, lists of genes were submitted to the Enrich software (accessed November 2022) [8].

Results

Morphology, immunohistochemical, and FISH analyses

Morphology, immunohistochemical, and FISH results are summarized in Figure 1.

Seven out of 35 undifferentiated BUC (20%) showed TROP-2 protein expression (2 cases with score 3+, 2 with score 2+, and 3 with score 1+), ranging from

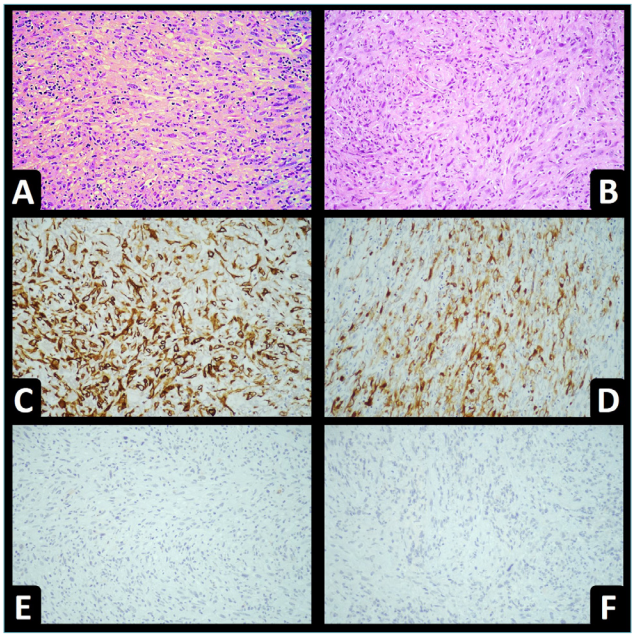


Figure 2. (A) Sarcomatoid urothelial bladder carcinoma (H&E; 200x). (B) Rhabdoid urothelial bladder carcinoma (H&E; 200x). (C) Sarcomatoid urothelial bladder carcinoma, TROP-2 diffuse immunoexpression. (D) Rhabdoid urothelial bladder carcinoma, NECTIN-4 diffuse immunoexpression. (E, F) Absence of TROP-2 and NECTIN-4 immunoexpression in sarcomatoid urothelial bladder carcinoma and rhabdoid urothelial bladder carcinoma respectively.

150 to 330 on the H-score (mean 240). NECTIN-4 was expressed in 10 cases (29%) of undifferentiated BUC. Seven cases (20%) co-expressed TROP-2 and NECTIN-4 (6 sarcomatoid and 1 rhabdoid). Three cases (2 sarcomatoid and 1 rhabdoid) were positive for NECTIN-4 but negative for TROP-2. Twenty-five cases (71%) were negative for both the markers (20 sarcomatoid and 5 rhabdoid). (Fig. 2)

HER-2 immunoexpression (score 1+, 2+, 3+) was observed in 14 cases (40%) (13 sarcomatoid and 1 rhabdoid). Only 5 cases (14%) scored 3+ showed *HER-2* amplification by FISH.

PD-L1 (SP263) scored positive for CPS (≥10) in 23/35 (66%) of cases with a diffuse positivity (> 30), whereas the PD-L1 (SP263) score using TPS revealed positive expression (> 5% of neoplastic cells) in 51% of cases. PD-L1 (22C3) scored with CPS was positive in 21 cases (60%).

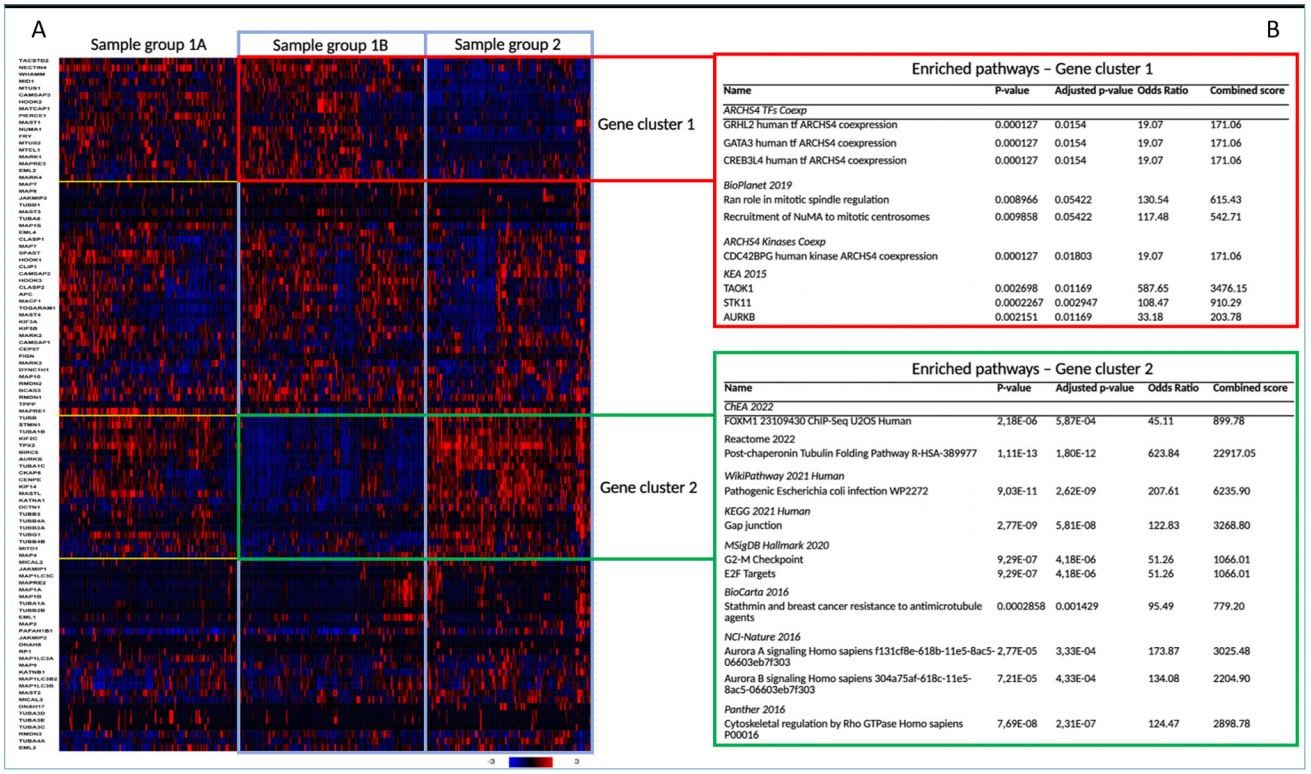
FGFR-2 or *FGFR-3* alterations were identified in 7 out of 35 cases (20%) using the FISH break-apart probe. Pan-NTRK1-2/3 immunoexpression was positive in 9 cases (26%).

Transcriptomic and pathway enrichment analysis

Trascriptomic findings are summarized in Figure 3A. Sample Groups 1B and 2 are characterized by the overexpression of genes of the *Gene cluster 1* and *Gene cluster 2* respectively. The third Group (Sample Group 1A) overexpressed genes of both the clusters (18 of Gene cluster 1 and 21 of Gene cluster 2). Other genes not included in the *Gene clusters 1* and *2* were variably expressed among the three sample groups.

After sending the list of clustered genes to the Enrich software, *Gene cluster 1* was enriched in genes associated with GRHL2, GATA3, CREB3L4, CDC42BPG expression with the protein kinase TAOK1, STK11, AURKB, with the RAN role in mitotic spindle regulation and with recruitment of NuMA to mitotic centrosomes ($P < 0.05$). *Gene cluster 2* was enriched in genes regulated by FOXM1, post-chaperon in tubulin folding, pathogenic E. coli infection, gap junction, G2-M checkpoint, E2F targets, stathmin and breast cancer resistance to antimicrotubule agents, Aurora A and B signaling and cytoskeletal regulation by Rho GTPase ($P < 0.05$) (Fig. 3B).

The top 200 genes positively correlated to NECTIN-4



Enriched pathways – Gene cluster 1

Name	P-value	Adjusted p-value	Odds Ratio	Combined score
ARCHS4 TFs Coexp				
GRHL2 human tf ARCHS4 coexpression	0.000127	0.0154	19.07	171.06
GATA3 human tf ARCHS4 coexpression	0.000127	0.0154	19.07	171.06
CREB3L4 human tf ARCHS4 coexpression	0.000127	0.0154	19.07	171.06
BioPlanet 2019				
Ran role in mitotic spindle regulation	0.008966	0.05422	130.54	615.43
Recruitment of NuMA to mitotic centrosomes	0.009858	0.05422	117.48	542.71
ARCHS4 Kinases Coexp				
CDC42BPG human kinase ARCHS4 coexpression	0.000127	0.01803	19.07	171.06
KEA 2015				
TAOK1	0.002698	0.01169	587.65	3476.15
STK11	0.0002267	0.002947	108.47	910.29
AURKB	0.002151	0.01169	33.18	203.78

Enriched pathways – Gene cluster 2

Name	P-value	Adjusted p-value	Odds Ratio	Combined score
ChEA 2022				
FOXM1 23109430 ChIP-Seq U2OS Human	2,18E-06	5,87E-04	45.11	899.78
Reactome 2022				
Post-chaperonin Tubulin Folding Pathway R-HSA-389977	1,11E-13	1,80E-12	623.84	22917.05
WikiPathway 2021 Human				
Pathogenic Escherichia coli infection WP2272	9,03E-11	2,62E-09	207.61	6235.90
KEGG 2021 Human				
Gap junction	2,77E-09	5,81E-08	122.83	3268.80
MSigDB Hallmark 2020				
G2-M Checkpoint	9,29E-07	4,18E-06	51.26	1066.01
E2F Targets	9,29E-07	4,18E-06	51.26	1066.01
BioCarta 2016				
Stathmin and breast cancer resistance to antimicrotubule agents	0.0002858	0.001429	95.49	779.20
NCI-Nature 2016				
Aurora A signaling Homo sapiens f131cf8e-618b-11e5-8ac5-06603eb7f303	2,77E-05	3,33E-04	173.87	3025.48
Aurora B signaling Homo sapiens 304a75af-618c-11e5-8ac5-06603eb7f303	7,21E-05	4,33E-04	134.08	2204.90
Panther 2016				
Cytoskeletal regulation by Rho GTPase Homo sapiens P00016	7,69E-08	2,31E-07	124.47	2898.78

Figure 3. (A) Heat map of 100 microtubule genes in addition to *NECTIN4* and *TACSTD2* of 411 bladder urothelial carcinoma cases extracted from the TCGA, PanCancer Atlas study by cBioPortal; clustering function included in cBioPortal applied to mRNA levels. (B) Enriched pathway of gene cluster 1 and gene cluster 2.

Table I. Enriched signaling pathways of top 200 genes positively correlated to the *NECTIN4* mRNA level in 411 bladder urothelial carcinoma cases of the TCGA, PanCancer Atlas study.

Databases and pathways*	P-value	Adjusted p-value	Odds ratio	Combined score
ENCODE and ChEA Consensus TFs from ChIP-X				
TP63 CHEA	3.4x10-04	3.2x10-02	2.19	17.46
ChEA 2022				
ESR1 26153859 ChIP-Seq MCF-7 Human BreastCancer	5.2x10-13	3.8x10-10	3.43	120.63
ESR2 21235772 ChIP-Seq MCF-7 Human	3.7x10-05	5.3x10-06	5.46	93.48
SOX2 20726797 ChIP-Seq SW620 Human	1.9x10-08	6.9x10-06	3.35	82.7
GATA3 24758297 ChIP-Seq MCF-7 Human	9.3x10-07	2.2x10-04	3.24	67.41
ESR1 26153859 ChIP-Seq T47D Human Breast Carcinoma	3.6x10-06	6.6x10-04	3.26	63.24
Reactome 2022				
GRB7 Events In ERBB2 Signaling R-HSA-1306955	9.7x10-06	1.7x10-03	150.75	1740.02
Fatty Acids R-HSA-211935	1.2x10-05	1.7x10-03	36.71	415.54
Kinase Perturbations from GEO up				
FGFR3 drug inhibition GDS5023	3.4x10-15	8.6x10-13	11.16	449.03
Kinase Perturbations from GEO down				
MAP3K7 knockdown GDS5023	3.5x10-10	8.9x10-08	8.68	248.82
SYK knockdown GDS3609	2.0x10-06	2.6x10-04	5.14	85

*underlined, databases provided by the Enrichr software.

($r > 0.5$, adjusted $P < 0.00002$) have been submitted to the software Enrich and the more significant results are reported in Table I. Factors and pathways enriched in NECTIN-4-related gene expression were TP63, ESR1 and ESR2 ChIP-Seq, SOX2 ChIP-Seq, GATA3 ChIP-Seq, GRB7 events in ERBB2 signaling, fatty acids, FGFR3 drug inhibition, MAP3K7 and SYK kinase knockdown ($P < 0.05$).

Discussion

We studied the expression of two microtubule proteins in a subset of undifferentiated BUC (sarcomatoid and rhabdoid) with the aim of finding a rationale for the use of drugs with strong activation against microtubule pathways in these less common cases. Indeed, TROP-2 and NECTIN-4 are the targets of new generation therapies using ADC drugs recently approved for the treatment of BUC [2]. Little is known about the expression of these two proteins in undifferentiated BUC. In our study TROP-2 and NECTIN-4 were not expressed in 71% of undifferentiated BUC. Conversely, in the literature, most of conventional BUC does express [2,4]. The cohort with expression of TROP-2 and NECTIN-4 (both or one of them) simultaneously showed at least one other molecular alteration. Overall, 11 of 35 (31%) undifferentiated BUC were silent for all biomarkers tested ("all negative" cases), providing no rationale for available targeted therapies. Based on these findings, we propose that the subset of "all-negative" undifferentiated BUC should be separated from those cases with expression of TROP-2 or NECTIN-4. The clinical impact on patients, when targeted therapies or ADCs are proposed, may be of high value.

Sacituzumab govitecan (SG) is an ADC targeting TROP-2 recently approved for treatment-refractory metastatic urothelial cancer. Tagawa et al. demonstrated SG to be an active drug with a manageable safety profile, with most common toxicities of neutropenia and diarrhea [3]. The authors showed that SG has a notable efficacy compared with historical controls in pretreated metastatic BUC that progressed on both prior platinum regimens. Of note, the authors did not study on tissue samples for the TROP-2 expression. The variability of TROP-2 expression between different bladder cancer subtypes, as well as after exposure to enfortumab vedotin (EV), prompted us to test the undifferentiated variants of BUC. Chu et al. evaluated NECTIN-4 by analyzing the mRNA expression in different molecular subtypes of bladder carcinoma and observed high expression in the luminal-subtypes with respect to others [2]. We cannot compare our data with those obtained with the antibody used in the study by Challita-Eid et al., because it was a mouse monoclonal antibody generated for the purpose of their study, and the clone is not commercially available [9].

Given the nature of the undifferentiated variants as a morphological pattern well known for harboring poor prognosticators, we elected to conduct simultaneous analysis of TROP-2 and NECTIN-4 in this context.

We integrated our study with the analysis of other markers important for proposed targeted therapies in BUC. In all cases positive for TROP-2 or NECTIN-4 we found the test positive for at least one other marker, while 11

cases were negative for all the markers (all-negative cases). Therefore, in our study, 31% of undifferentiated BUC, the 11 all-negative cases, hypothetically lack the rationale for currently available targeted therapies.

Immunoexpression of HER-2 is observed in a subset of sarcomatoid and rhabdoid BUC, although most cases did not harbor *HER-2* gene amplification. This data is comparable to what was observed in conventional BUC cases [10]. However, a HER-2 low-content may have impact on the use of new generation drugs which does have effect using the protein bridges of the membranes of neoplastic cells. The positive immunohistochemical value may be observed in cases with and without both TROP-2 and NECTIN-4 protein expression even if the amplification of HER-2 is present only in cases positive for TROP-2 and NECTIN-4.

Again, of note, a significant subset of sarcomatoid and rhabdoid bladder carcinoma does show diffuse expression of CPS PD-L1 by both sp263 and 22C3 clones, and also TPS PD-L1 by sp263. PD-L1 expression was independently associated with other positive immunoexpression and using different clones (sp263 and 22C3) we confirm their good concordance (*h-index* 0.86) as previously described [11].

FGFR was altered (gains or losses) in a minority of cases, such as pan-NTRK expression. These groups are observed in both TROP-2/NECTIN-4 with negative or positive patterns.

We detailed this differentiation using immunohistochemistry and cytogenetics, and we matched the public repository of molecular genomic findings from the International cBioPortal and found such a difference along the public dataset of molecules of the microtubule pathways. The previous match reinforces our findings using tissue analysis, given the visibility of the warm and cold pathways of 100 molecules related to microtubules along the dataset. The heterogeneity of immunophenotypic and molecular biological expression observed in our study cohort is similar to the molecular heterogeneity extracted from public international repositories.

At the transcriptomic level, three groups of mRNA levels were observed. Group 1A showed overexpression of 18 genes of the Gene cluster 1 and 21 genes of the Gene cluster 2, whereas group 1B overexpressed only genes of the Gene cluster 1 and group 2 overexpressed only genes of the Gene cluster 2. Moreover, other genes for microtubules were variably expressed among the three groups. These findings do show that expression of targets of ADC drugs may have potential impact to the efficacy of therapies, based on the magnitude of presence of microtubules, either at percentages of neoplastic cells with immunoexpression or for intensity. These patterns are reflected and replaced by genomic analysis where clusterization is clearly seen. In other words, potential resistance of such use of drugs or improvements of major magnitude of activation of the drugs may be answered by simply grading or scoring the immunohistochemical, FISH or mRNA clusterization. At the single gene level, gene cluster 1 was enriched with genes associated with GRHL2, GATA3, CREB3L4, CDC42BPG expression and the protein kinase TAOK1, STK11, AURKB, RAN role in mitotic spindle regulation, recruitment of NuMA to mitotic centrosomes. Gene cluster 2 was enriched with genes associated with cell cycle and regulated by FOXM1, pathogenic *E. coli* infection, gap junction, stathmin and breast cancer resistance to anti-microtubule agents, Aurora A and B signaling and cytoskeletal regulation by Rho GTPase.

The discussion of the connection between the ADC (antibody-drug conjugate) target NECTIN-4 and several other cellular factors and pathways such as TP63, estrogen signaling, SOX2, GATA3, ERBB2 signaling, FGFR3 drug inhibition, MAP3K7, and SYK kinases control pathways, points toward a complex interplay of molecular interactions and signaling pathways involved in the regulation of cellular functions, particularly those associated with bladder urothelial carcinoma (BUC). Understanding the molecular interactions and pathways involved in the regulation of NECTIN-4 in conjunction with other factors and signaling pathways is crucial to design effective treatment strategies for BUC. Some of these significant evidences have already been tested for the differential diagnosis of poorly differentiated urothelial carcinoma [12]. For instance, a TP63-related expression signature is known to be associated with the squamous phenotype of different cancer types and the ΔN variant of p63, p40, together with GATA-3 and uroplakin II, have been shown useful in the diagnosis and prognosis of muscle-invasive urothelial carcinoma [13]. Moreover, SOX2 has been found to be a marker of stem-like tumor cells in BUC [14] and promoter of stemness malignancy properties in urothelial cells [15]. Additionally, a recent study suggests that GATA-3 could represent a biomarker of cell differentiation rather than of cancer cell malignancy [16]. Considering these molecular connections and pathways, a synthetic lethality approach could be employed to selectively target the vulnerabilities of cancer cells with specific genetic alterations. This strategy would involve exploiting the specific dependencies or vulnerabilities created by the interaction between NECTIN-4 and the aforementioned factors and pathways.

The current retrospective study focuses on the biological patterns of expression in bladder urothelial carcinoma (BUC) with an emphasis on two microtubules proteins TROP-2 and NECTIN-4. Although the study cannot include information on the response to therapy, it can still shed light on the distinct biological

patterns of expression associated with the presence or absence of these biomarkers.
Moreover, at the technical level, we designed the study with the aim to assess biomarkers on whole tissue blocks on pure undifferentiated BUC rather than small tissue samples like tissue microarrays or biopsies.

Conclusions

The simultaneous evaluation of various biomarkers like TROP-2, NECTIN-4 in addition to PD-L1, *HER-2*/HER-2, *FGFR*, and pan-NTRK in bladder carcinoma has shown promising implications at the clinical level. This evaluation appears to have utility for both prognostication and predictiveness for precision therapies such as antibody-drug conjugates (ADC). In this study, 32% of sarcomatoid urothelial carcinoma were silent for all biomarkers (TROP-2/NECTIN-4 and others), termed as the "all negative" pattern, whereas only 11% did show activation of all biomarkers (TROP-2/NECTIN-4 plus others). Along cases with rhabdoid dedifferentiation, 14% cases showed activation of all biomarkers, whereas 28% were "all negative". The remaining cases did show heterogeneous activation. Such distinctions could impact the effectiveness of ADC drugs or other targeted therapies in undifferentiated BUC.

Conflict of interest statement

The authors declare no conflict of interest.

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Ethical consideration

Approved by the ethics committee procedure code PRIHTA-2014-00000453

Authors' contributions

MB: wrote the paper, performed the analysis; SG: wrote the paper, performed the analysis; GM: performed the analysis; GS: collected the data; CC: collected the data; EM: performed the analysis; SF: collected the data; AC: performed the analysis; GM: collected the data; AC: collected the data; AV: collected the data; AA: collected the data; MT: collected the data; FP: collected the data; IMH: other contribution; AE: collected the data; SA: collected the data; MM: other contribution; LB: collected the data; LC: wrote the paper; SB: conceived and designed the analysis.

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